PROTOCOL BACONTRACEPTION UNIVERSITAS INDONESIA

PART TWO : PROTOCOL WET LAB IN INDONESIA LABORATORY

Preparation Medium Agar and Broth for Bacteria

A. Preparation Nutrient Agar and Broth

Ingredients and Tools :

a. Nutrient Agar and Nutrient Broth
b. Aquabidest
c. Weight Measurement
d. Autoclave
e. Plate and glass bottles or glass tube

Step :

a. Weighting 400 miligrams nutrient broth and 2700 miligrams nutrient agar.
b. Throwing each (nurient broth and agar) into different steril glass.
c. Adding 120 ml aquabidest for steril glass containing nurient broth and 50 ml aquabidest for steril glass containing nutrient agar.
d. Stirring and Boiling these glasses until until all the ingredients dissolve completely, then autoclave these at 121^{o}C for about 30 minutes.
e. Next, Pouring about 30 ml nutrient agar into plat. Otherwise, Pouring nutrient broth in glass tube . (We did this step in Biosafety Cabin and aceptis procedural)
f. Place it overnight in the incubator for medium
g. Then take it in the next day for ensuring there is contamination or no

B. Preparation Mueller Hinton

a. Weighting 3800 miligrams nutrient agar.
b. Throwing each (nurient broth and agar) into different steril glass.
c. Adding 100 ml aquabidest for steril glass containing nurient broth and 50 ml aquabidest for steril glass containing nutrient agar.
d. Stirring and Boiling these glasses until until all the ingredients dissolve completely, then autoclave these at 121^{o}C for about 30 minutes.
e. Next, Pouring about 30 ml nutrient agar into plat. Otherwise, Pouring nutrient broth in glass tube . (We did this step in Biosafety Cabin and aceptis procedural)
f. Place it overnight in the incubator for medium
g. Then take it in the next day for ensuring there is contamination or no

Making Frozen Stock for Bacteria

Isolation

A. Genom Isolation

Ingredients and Tools :

a. Nutrient Agar and Nutrient Broth
b. Aquabidest
c. Weight Measurement
d. Autoclave
e. Plate and glass bottles or glass tube

Step :

a. Prepare Nutrient Broth in tube.
b. Take single coloni of Bacillus subtilis from nutrient agar plate which is growth inside the incubator at 32^{O} by ose or an other steril stick
c. Inoculate this into Nutrient Broth
d. Shake it for about 3-4 hours in shaker incubator at 32^{O}c
e. Centrifuge the culture at 32^{O}C, 3 minutes, 10000xgram.
f. Throw away the supernatan by pipeting it slowly. Finally, we get the cell
g. Add 557 ul STET buffer and vortex it
h. Add 10 ul lisozim 10 mg/ml; 30 ul SDS 10% ; and 30 mg/ml,....
i. Incubate it at 37oC incubator for about 60 minutes
j. Add 65ul NaCl 4 M and 80 ul CTAB buffer. Vortex it.
k. Incubate at 65oC for 30-60 minutes
l. Add 650 ul chloroform and isoamilalcohol mixture, then centrifuge it at 20^{o}C for 2 minutes, 10.000xg. Finally, we get 3 layers (supernatan-cell-chloroform liquid)
m. Pipet the supernatan and pour it in an other tube. Centrifuge again it at 20^{o}C for 2 minutes, 10.000xg.
n. Add 400 ul Isopropamol cold. Then, Invert the tube 3-4 times slowly.
o. Centrifuge at 20oC for about 3 minutes, 10.000xg.
p. Throw away supernatan and we get Ethanol 70%. Then, Invert the tube 3-4 times slowly.
q. Centrifuge at 20oC, 10.000xg for about 2 minutes. Throw away the supernatan.
r. Dry it at room temperature for about 2 hours
s. Add 25 ul destilate water or TE . In last, incubate it at 37oC for 15 minutes.

t. DNA genomic in destilate water is already to electrophoresis (for checking the amount of base)

B. Plasmid Isolation UsingMiniprep from The Product of Bioneer

a. Pick up a single colony from fresh cultured Luria Bertani agar plate contain antibiotics
b. Inoculate the cell into 1-5 ml of fresh LB liquid media with shaking for 16 houurs at 37oC.
c. Collect the E.coli cells by centrifugation at 12.000 rpm for 2 minutes and completely remove of the media by pipetting.
d. Add 250 ul Buffer one to collected cells and completely resuspend by vortexing.
e. Add 250 ul of buffer 2 and mix by inverting the tube 5 times gently/
f. Add 700 ul buffer 4 to the DNA binding column tube and centrifuge 12.000 rpm for 1 minutes. Pour off the flow-through and re-assemble the DNA binding filter column with the 2 ml collection tube
g. Dry

Electrophoresis

A. Electrophoresis Agarose gel

a. Weighting 2 gram agarose
b. Throw it in the erlenmeyer or an other glass. Add 25 ml TAE buffer 0,5x.
c. Dissolve it well by microwave.
d. After that, wait it until the temperature reach at 45-55^{o}C. Pour it into the place of agar in electrophoresis device. Wait the agar change to be more hard and ready for loading the DNA.
e. Load the mixture 2ul DNA and 1 ul loading dye for DNA electrophoresis (we us it from bioneer product) into well of agar.i
f. Load the 100 bp marker from bioneer product in the last well of agar.
g. Power on the electrophoresis device and set this device for 100 voltage for 25 minutes
h. Stainning the gel using gel red for 10 minutes
i. De-stainning the gel to clear for 10 minutes .
j. We can find the result by gel doc device with uv light

Polimerase Chain Reaction

A. Polimerase Chain Reaction for Confirmation by Accu Power Premix Bioneer

- Add template DNA and primers to AccuPower® PCR tube.

Component \ Reaction size	20 µl reaction	50 µl reaction
Top DNA polymerase	1 U	2.5 U
Each: dNTP (dATP, dCTP, dGTP, dTTP)	250 µM	250 µM
Tris-HCl (pH 9.0)	10 mM	10 mM
KCl	30 mM	30 mM
$MgCl_2$	1.5 mM	1.5 mM
Stabilizer and tracking dye		

- Add distilled water to AccuPower® PCR tubes to a total volume of 20 µl or 50 µl.
- Dissolve the lyophilized blue pellet by vortexing, and briefly spin down.
- Add mineral oil. (This step is unnecessary when using a thermal cycler with top heating.)
- Perform PCR of samples.
- Load samples on agarose gel without adding a loading-dye mixture, and perform electrophoresis.

B. Polimerase Chain Reacton with HotStart.

Making competent cell

A. Making Top 10 Competent Cell Using CCMB80 Buffer

CCMB80 Buffer

- 10 mM KOAc pH 7.0 (10 ml of a 1M stock/L)
- 80 mM $CaCl_2.2H_2O$ (11.8 g/L)
- 20 mM $MnCl_2.4H_2O$ (4.0 g/L)
- 10 mM $MgCl_2.6H_2O$ (2.0 g/L)
- 10% glycerol (100 ml/L)
- adjust pH DOWN to 6.4 with 0.1N HCl if necessary
- adjusting pH up will precipitate manganese dioxide from Mn containing solutions.
- sterile filter and store at 4°C

Preparing seed stocks

- Streak TOP10 cells on Luria Bertani agar and grow for single colonies at 23°C
- Pick single colonies into 2 ml of Luria Bertani agar and shake overnight at 23°C
- Add glycerol to 15%
- Aliquot 1 ml samples to microtube
- Place tubes into a zip lock bag, immerse bag into a dry ice/ethanol bath for 5 minutes
- This step may not be necessary

- Place in -80°C freezer indefinitely.

Preparing competent cells

- Inoculate 250 ml of Luria Bertani Broth with 1 ml vial of seed stock and grow at 20°C to an OD600nm of 0.3
- This takes approximately 16 hours.
- Centrifuge at 3000rpm at 4°C for 10 minutes
- Discard supernatant by pouring out slowly and pipeting remaining supernatent
- Gently resuspend in 80 ml of ice cold CCMB80 buffer.
- Incubate on ice 20 minutes
- Centrifuge again at 4°C and discard supernatant as described above.
- Resuspend in 10 ml of ice cold CCMB80 buffer.
- Test OD of a mixture of 200 µl SOC and 50 µl of the resuspended cells.
- Add chilled CCMB80 to yield a final OD of 1.0-1.5 by elisa reader.
- Aliquot to chilled screw top 2 ml vials .
- Store at -80°C indefinitely.

B. Making DH5alpha Competent cell

a. Taking fresh single coloni of E.coli DH5alpha and growing this in Luria Broth. Then, Shake this overnight in incubator shaker at 37oC.
b. Pipet 0,4 ml E.coli Top 10 culture overnight and pour it into falcon. Next, this falcon is shaked again in shaker incubator for 3-4 hours at 37oC.
c. Pipet 1,5 culture from step 2 into new sterile mikrotube and centrifuge this to get the pellete at 5000 rpm for 1 minute.
d. Add 1 ml 0,1 M CaCl2 cold for each mikrotube and vortex
a. Placing this mikrotube in the box which is filled by ice cube and wait for 15 minutes.
b. Centrifuge to get pellete at 5000 rpm for 1 minutes (by throwing away the supernatan)
c. Add 200 ul CaCl2 cold liqud and wait this in ice cube for 10 minutes.

Transformation

a. Pipet 50 ul E.coli DH5alpha competent cell and into sterile microtube.
b. Add 1 ul sample (we use the recombination BP) and mix it slowly.
c. Incubate in ice cube for 30 minutes.
d. Heat shock at 42oC for 90 seconds.

e. Incubate in ice cube immediately for 2 minutes.
f. Add 450 ul SOC medium
g. Incubate at 37oC for one hour and shacked at 200 rpm.
h. Preparing some LB-canamycin agar plates. After that, give 20 ul and 100 ul the result of transformation in different plates.
i. Incubate overnight at 37oC

Digestion

A. Double Digest

a. Calculate all ingredients for double digest. We usually use total volume 25 ul rnx.

Total Reaction Volume	**Restriction Enzyme***	**DNA**	**10X NEBuffer**
10 µl rnx**	1 unit	0.1 µg	1 µl
25 µl rnx	5 units	0.5 µg	2.5 µl
50 µl rnx	10 units	1 µg	5 µl

b. Incubate for 1 hour in 37^{o}C
c. Heat kill in 80^{o}C for 20 minutes

B. Digestion PSB1C3 linier

a. Enzyme Master Mix for Plasmid Backbone (25ul total, for 5 rxns)
   - 5 ul NEB Buffer 2
   - 0.5 ul BSA
   - 0.5 ul EcoRI-HF
   - 0.5 ul PstI
   - 0.5 ul DpnI (Used to digest any template DNA from production)
   - 18 ul dH20

b. Digest Plasmid Backbone
   - Add 4 ul linearized plasmid backbone (25ng/ul for 100ng total)
   - Add 4 ul of Enzyme Master Mix
   - Digest 37C/30 min, heat kill 80C/20 min

Ligation

Ligation with PSB1C3 linier

a. Add 2ul of digested plasmid backbone (25 ng)
b. Add 0,5 ul EcoRI-HF digested fragment (< 3 ul)
c. Add 0,5 ul PstI digested fragment (< 3 ul)
d. Add 1 ul T4 DNA ligase buffer. **Note:** Do not use quick ligase
e. Add 0.5 ul T4 DNA ligase
f. Add water to 10 ul
g. Ligate 16C/30 min, heat kill 80C/20 min
h. Transform with 1-2 ul of product

Ligation

a. Calculate the component for ligation.

COMPONENT	**20 µl REACTION**
10X T4 DNA Ligase Buffer*	2 µl
Vector DNA	50 – 100 ng
Insert DNA	Should be calculated (insert mass in ng = 3 x (insert length/vector length) x vector mass)
Nuclease-free water	to 20 µl
T4 DNA Ligase	1 µl

b. * *The T4 DNA Ligase Buffer should be thawed and resuspended at room temperature.*
c. Gently mix the reaction by pipetting up and down and microfuge briefly.
d. For cohesive (sticky) ends, incubate at 16°C overnight or room temperature for 10 minutes.
e. For blunt ends or single base overhangs, incubate at 16°C overnight or room temperature for 2 hours*(alternatively, high concentration T4 DNA Ligase can be used in a 10 minute ligation).*
f. Heat inactivate at 65°C for 10 minutes.
g. Chill on ice and transform 1-5 µl of the reaction into 50 µl competent cells.

Gibson Assambley

a. Resuspensi gBlocks

• Centrifuge the tube for 3−5 sec at a minimum of 3000 x g to pellet the material to the bottom of the tube.

• Add TE to the tube for your desired final concentration

• Briefly vortex and centrifuge

b. Gibson Assembly Master Mix

• The mass of each fragment can be measured using the **NanoDrop instrument**, absorbance at 260 nm or estimated from agarose gel electrophoresis followed by ethidium bromide staining.

• * Optimized cloning efficiency is 50–100 ng of vectors with 2–3 fold of excess inserts. Use 5 times more of inserts if size is less than 200 bps. Total volume of unpurified PCR fragments in Gibson Assembly reaction should not exceed 20%. ** Control reagents are provided for 5 experiments. *** If greater numbers of fragments are assembled, additional Gibson Assembly Master Mix may be required.

• Incubate samples in a thermocycler at 50°C for 15 minutes when 2 or 3 fragments are being assembled or 60 minutes when 4-6 fragments are being assembled. Following incubation, store samples on ice or at -20°C for subsequent transformation.

Note: Extended incubation up to 60 minutes may help to improve assembly efficiency in some cases (for further details see FAQ section).

Bacillus subtilis Electrophorasi

a. Making electrocompetent cells

- Dilute an overnight culture of Bacillus subtilis 16-fold in growth medium and grow at 37^{o}C to an O.D.$_{600}$ of 0,85-0,95
- Cool the cells on ice-water for 10 minutes and harvest by centrifugation at 4^{o}C and 5000xg for 5 minutes
- Wash cells four times in ice-cold electroporation medium
- Suspend the cells in 1/40 of the culture volume of the electroporation solution with a cell conentration of 1-1,3x10^{10} cfu/ml

b. Electroporation of cells :

- Add 1 ul plasma DNA to 60 ul competent cell. Homogenizie by gently mixing with pipette several times. Transfer mixture into a prichilled cuvette. Incubate for 1-1,5 minutes.
- Electroporation

- Immidiately add 1 ml outgrowth medium and incubate for 3 hours at 37^{o}C
- Plate onto selective LB agar plates and incubate overnight at 37^{o}C

Purification

a. Purification QIAEX

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel
2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).
3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.
4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).
5. Add 1 gel volume of isopropanol to the sample and mix
6. Place a QIAquick spin column in a provided 2 ml collection tube. 7. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min
7. Discard flow-through and place QIAquick column back in the same collection tube.
8. (Optional): Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min
9. To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.
10. Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at ≥10,000 x g (~13,000 rpm).
11. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.
12. To elute DNA, add 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or H2O to the center of the QIAquick membrane and centrifuge the column for 1 min at maximum speed. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min